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Production of Pediocin AcH by *Lactobacillus plantarum* WHE 92 Isolated from Cheese

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Among 1,962 bacterial isolates from a smear-surface soft cheese (Munster cheese) screened for activity against *Listeria monocytogenes*, six produced antilisterial compounds other than organic acids. The bacterial strain WHE 92, which displayed the strongest antilisterial effect, was identified at the DNA level as *Lactobacillus plantarum*. The proteinaceous nature, narrow inhibitory spectrum, and bactericidal mode of action of the antilisterial compound produced by this bacterium suggested that it was a bacteriocin. Purification to homogeneity and sequencing of this bacteriocin showed that it was a 4.6-kDa, 44-amino-acid peptide, the primary structure of which was identical to that of pediocin AcH produced by different *Pediococcus acidilactici* strains. We report the first case of the same bacteriocin appearing naturally with bacteria of different genera. Whereas the production of pediocin AcH from *P. acidilactici* H was considerably reduced when the final pH of the medium exceeded 5.0, no reduction in the production of pediocin AcH from *L. plantarum* WHE 92 was observed when the pH of the medium was up to 6.0. This fact is important from an industrial angle. As the pH of dairy products is often higher than 5.0, *L. plantarum* WHE 92, which develops particularly well in cheeses, could constitute an effective means of biological combat against *L. monocytogenes* in this type of foodstuff.

A microbiological study of smear-surface soft cheeses (Munster type), carried out in 1992 with 357 samples prepared from pasteurized milk and 115 samples prepared from raw milk, has shown high-level contamination of these cheeses with *Listeria monocytogenes*. This bacterium was found in almost 60% of cheeses prepared from pasteurized milk (with levels higher than 10^5 CFU/g in 6% of the samples) and in 80% of cheeses prepared from raw milk (with levels higher than 10^5 CFU/g in 20% of the samples) (10). This result was not surprising, since the rind of these cheeses provides an excellent culture medium for *L. monocytogenes*. In other respects, the repeated handlings and washes carried out during ripening constitute an effective mode of contamination. During the same study (10), it was also shown, more unexpectedly, that a group of 74 Munster cheeses of farm origin (made with raw milk), ripened in the same cellar as preceding groups of industrial manufacture, demonstrated a much lower level of contamination with *L. monocytogenes*. Only 25% of these cheeses of farm origin were contaminated, at levels generally lower than 10^2 CFU/g and never higher than 10^4 CFU/g (9). Such a result could possibly be explained by the presence of lactic acid bacteria, capable of producing antilisterial bacteriocins, in the rind of these cheeses. To date, this kind of bacterium has not been isolated from the rind of smear-surface soft cheeses. However several lactic acid bacteria, capable of producing antilisterial bacteriocins, especially of the *Lactobacillus* genus (27), have been found in other dairy products, meat products, and vegetables (12, 19, 21, 27, 29, 31, 32, 40, 54). An antagonistic bacterial strain capable of developing harmoniously in the cheese and producing a proteinaceous antilisterial substance which would be rapidly broken down after human ingestion would be an interesting means of biological combat against *L. monocytogenes.*

The purpose of the present study was, firstly, to research such lactic acid bacteria in the group of farm Munster cheeses, and, secondly, once the bacteriocin was found, to characterize it, purify it, and determine its chemical structure in order to identify it.

MATERIALS AND METHODS

Bacterial strains and cultures. The bacterial strains used in this study to determine the spectra of the antibacterial substances are described in Table 1. *L. monocytogenes* 4d was chosen as the indicator strain to demonstrate and to measure bacteriocin activity.

Lactobacillus plantarum 1904, used in DNA homology experiments, was obtained from the Czechoslovak Collection of Microorganisms, Brno, Czech Republic. *Pediococcus acidilactici* H, a pediocin AcH producer, was supplied by N. Kalchayanand, University of Wyoming, Laramie.

All cultures were maintained as frozen stocks held at −80°C in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) with 30% glycerol. Throughout the experiments, strains were subcultured every 2 weeks on strain preservation agar slants (Diagnostics Pasteur, Marnes-La-Coquette, France) and kept at 4°C. Before experimental use, cultures were propagated twice in broth for 18 to 24 h. The following cultures were grown in the indicated media: lactobacilli, pediococci, and enterococci, MRS (de Man, Rogosa, and Sharpe) broth (Oxoid Ltd., Basingstoke, Hampshire, England); *Lactococcus lactis*, M_{17} broth (Merck, Darmstadt, Germany); *Clostridium sporogenes*, TGY broth (Diagnostics Pasteur, Marnes-La-Coquette, France); *Mycobacterium smegmatis*, Dubos Tween 80 broth (Diagnostics Pasteur); *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella infantis*, brain heart infusion broth (Difco Laboratories); and other target strains, TSYE (tryptone soy broth [Biokar Diagnostics, Beauvais, France] containing 0.6% yeast extract [Biokar Diagnostics]) broth. The incubation temperature was 37°C, except for *Brevibacterium linens*, *L. lactis*, *Micrococcus sedentarius*, and *M. smegmatis* (30°C) and *P. aeruginosa* (25°C). Incubation for *Clostridium sporogenes* was performed under anaerobic conditions.

Isolation of antilisterial lactic acid bacteria and activity assays. The search for anti-*L. monocytogenes* lactic acid bacteria was carried out by using farm-produced Munster cheeses. Ten grams of food sample was homogenized in a stomacher blender for 2 min with 90 ml of TSYE broth, 10-fold serially diluted, and plated on MRS agar (7) or M_{17} agar (53).

Antibacterial activity was detected by the direct detection method described by Barefoot and Klaenhammer (2), using *Lactobacillus* agar AOAC (Difco Labo-

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TABLE 1. Sensitivities of various indicator bacteria against the bacteriocin in this study

Indicator species	Strain	Source[a]	Inhibition zone diam (mm)
Listeria monocytogenes	1/2a	LC	10
Listeria monocytogenes	1/2b	LC	10
Listeria monocytogenes	1/2c	CIP	12
Listeria monocytogenes	3a	LC	8
Listeria monocytogenes	3b	LC	10
Listeria monocytogenes	4b	LC	12
Listeria monocytogenes	4d	LC	14
Listeria innocua	6a	LC	10
Listeria innocua	6b	LC	8
Listeria seeligeri	26	LC	12
Enterococcus faecium	81	LC	8
Enterococcus faecium	54.32	CIP	0
Enterococcus hirae	10541	ATCC	6
Lactococcus lactis	148	CNRZ	0
Lactococcus lactis	11454	ATCC	0
Lactobacillus curvatus	25601	ATCC	6
Lactobacillus plantarum	WHE 92	This study	0
Lactobacillus brevis	14869	ATCC	0
Lactobacillus paracasei	25598	ATCC	0
Pediococcus pentosaceus	25744	ATCC	6
Pediococcus acidilactici	H	NK	0
Brevibacterium linens	SR3	LN	0
Micrococcus sedentarius	33	LC	8
Staphylococcus xylosus	57	LC	16
Staphylococcus aureus	9144	ATCC	0
Clostridium sporogenes	79.39	CIP	0
Bacillus cereus	78.3	CIP	0
Bacillus cereus	504b	LC	11
Bacillus subtilis	6633	ATCC	0
Bacillus subtilis	9372	ATCC	0
Bacillus thuringiensis	10792	ATCC	0
Bacillus pumilus	4041b	LC	0
Mycobacterium smegmatis	73.26	CIP	0
Escherichia coli	10536	ATCC	0
Pseudomonas aeruginosa	A22	CIP	0
Salmonella infantis	8	LC	0

[a] Abbreviations: ATCC, American Type Culture Collection (Rockville, Md.); CIP, Collection de l'Institut Pasteur (Paris, France); CNRZ, Centre National de Recherches Zootechniques (Jouy-en-Josas, France); LC, laboratory collection; LN, Lactolabo (Nancy, France); NK, N. Kalchayanand (University of Wyoming).

ratories) seeded with an overnight culture of *L. monocytogenes* 4d (made in TSYE broth at 37°C) at a level of ca. 10^6 CFU/ml as the overlayer. After overnight incubation, bacterial colonies found positive for antilisterial activity were picked up through the inhibition zone, streaked for single-colony isolation, and further tested. Isolates were inoculated in MRS broth to yield an initial concentration of ca. 10^3 CFU/ml and incubated for 18 h at 37°C. After centrifugation (2,700 × *g* for 10 min), the supernatant was adjusted to pH 6 with 1.0 M NaOH (Merck) and filter sterilized (0.45-μm-pore-size cellulose ester filter; Costar). Antilisterial activity of the obtained cell-free filtrate (culture extract) was tested by the well diffusion assay (deferred method) described by Tagg and McGiven (52), with a double layer consisting of MRS agar and *Lactobacillus* agar seeded with *L. monocytogenes* 4d as for the direct method. To quantitate inhibitory activity, the diameter of the inhibition zone (in millimeters) was measured. For strong activities, the titer (expressed in activity units [AU] per milliliter), corresponding to the reciprocal of the highest dilution showing a distinct inhibition zone of the indicator lawn, was determined (34).

Taxonomical tools. Sugar fermentation abilities were tested at 37°C with the API 50CHL system (BioMérieux). The production of gas from glucose was tested with MRS broth lacking beef extract, and the production of gas from gluconate was determined with MRS broth lacking beef extract and glucose but containing gluconate (10 g/liter). The gas produced was trapped in Durham tubes. Characterization at the DNA level was performed by Southern-type hybridization with an *L. plantarum pyrDFE* probe as described by Bringel et al. (5), with the modification that high-stringency conditions were used (hybridization was performed at 68°C instead of 50°C). Genomic DNAs were extracted as described by Vanderslice et al. (58).

Bacteriocin presence in *L. plantarum* WHE 92 culture extract. (i) Susceptibility to proteolytic and other enzymes. The culture extract of the selected strain was treated with several enzymes: trypsin (14 U/ml; Sigma), α-chymotrypsin (350 U/ml; Sigma), pronase E (8.8 U/ml; Sigma), ficin (0.5 U/ml; Sigma), pepsin (91 U/ml; Sigma), lipase (4.2 U/ml; Sigma), α-amylase (0.6 U/ml; Sigma), and catalase (2,600 U/ml; Merck). All samples were adjusted to pH 7 with 1.0 M NaOH (Merck), except the one treated with pepsin (pH 2 [adjusted with 37% HCl; Merck]), filter sterilized (0.45-μm-pore-size cellulose ester filter; Costar), and held for 1 h at 37°C when treated with pronase E, pepsin, and lipase and at 25°C when treated with other enzymes. The residual activity of treated and control samples was determined by measuring the diameters of the inhibition zones in the well diffusion assay.

(ii) Activity spectrum. By the well diffusion assay described above for *L. monocytogenes* 4d, the antagonistic effect of the antibacterial substance against various gram-positive and gram-negative bacterial genera was tested (Table 1). The double layer consisted of the appropriate soft agar (1% agar) seeded with the target strain as described for *L. monocytogenes* 4d and overlaid on MRS agar. The plates were incubated at the optimum growth temperature of the target strain and examined after 18 to 24 h.

(iii) Mode of action. The culture extract with the antibacterial substance was inoculated with an overnight culture of *L. monocytogenes* 4d (made in TSYE broth at 37°C) at a ratio of 1:10 in order to yield an initial bacterial level of ca. 10^7 CFU/ml. After incubation of the bacterial suspension at 37°C, viable counts of *L. monocytogenes* were determined on TSYE agar plates at various time intervals.

Morphological changes in *L. monocytogenes* after treatment with the antibacterial substance were observed with a scanning electron microscope (Stereoscan 240; Cambridge Instruments). Control and treated (13,500 AU/ml) bacterial cells were prepared as described by Kato et al. (25), with the slight modification that the slide glasses were dried at 55°C for 30 min. Prior to observations, slide glasses with the fixed cells were ion spatter coated with gold (MED 010; Balzers Union). Photographs were printed on a Sony apparatus (Mavigraph, UP-7000P).

Effect of pH on bacteriocin production in liquid media. The influence of final culture pH on bacteriocin production was investigated according to the method described by Biswas et al. (4), with TGE broth for *P. acidilactici* H, MRS broth for *L. plantarum* WHE 92, and incubation at 37°C. Cultures were carried out in a 2-liter fermentor (Chemap AG) with an automatic controller (CBC5) of temperature, pH (by the addition of sterile 1.0 M NaOH), agitation speed (500 rpm), and foam level (by the addition of sterile 10% silicon oil [Merck]). The fermentor was inoculated with a late-exponential-growth-phase culture in order to yield an initial population of ca. 10^3 CFU/ml. The initial pH of the culture broths was 6.5. After incubation, first it was allowed to drop to a desired level (i.e., final pH of 6.0, 5.5, 5.0, 4.5, or 4.0) and then it was maintained by the fermentor's automated pH control. In the case of pH 6.5 as final pH, the initial pH was the same as the final pH and the pH was thus continuously maintained at this value. After 18 h of incubation, bacterial counts at late exponential growth phase were determined on TSYE agar plates and bacteriocin production was monitored by titration.

Bacteriocin purification. The bacteriocin was purified from 2-liter cultures of *L. plantarum* WHE 92 grown in MRS broth at 37°C to late exponential phase. Purification was performed according to the four-step procedure described by Nissen-Meyer et al. (42): (i) ammonium sulfate precipitation, (ii) purification by passage through a cation exchanger (S-Sepharose, Sigma), and (iii) reversed stationary-phase (octyl-Sepharose [CL-4B; Sigma]) and (iv) stationary-phase (C_2-C_{18} [PepRPC HR 5/5; Pharmacia-LKB]) chromatography.

When applied to the C_2-C_{18} column, the bacteriocin was eluted, at a flow rate of 1 ml/min, with a gradient of H_2O-isopropanol mobile phase containing 0.1% trifluoroacetic acid (Waters 600E pump; Millipore) and detected at 280 nm (Waters 991 detector; Millipore). After the first injection, fractions of 1 ml eluting from the column (gradient of H_2O-isopropanol containing 0.1% trifluoroacetic acid [from 0 to 5 min, 90:10, vol/vol; from 5 to 45 min, from 90:10 to 60:40, vol/vol]) were collected. Those corresponding to the bacteriocin peak (recognized by titration of the antibacterial activity) were diluted four- to fivefold with H_2O containing 0.1% trifluoroacetic acid and rechromatographed on the same stationary phase column. The bacteriocin was then eluted with a gradient of H_2O-isopropanol containing 0.1% trifluoroacetic acid: from 0 to 5 min, 90:10, vol/vol; from 5 to 15 min, from 90:10 to 75:25, vol/vol; from 15 to 40 min, from 75:25 to 65:35, vol/vol. The UV absorption spectrum of the fraction containing the purified bacteriocin was determined (Uvikon 930 spectrophotometer; Kontron Instruments). The latter was stored at −20°C in an H_2O-isopropanol mixture (50:50, vol/vol) containing 0.1% trifluoroacetic acid.

Amino acid sequence and mass analyses. The amino acid sequence of the bacteriocin was determined by Edman degradation on a liquid-phase automatic sequence analyzer (model 473A; Applied Biosystems). Disulfide bonds were localized by hydrolysis of the peptide with a Lys-C endoproteinase. The molecular mass of the purified sample was determined by an electrospray ionization mass spectrometer coupled to a quadripolar analyzer (Bio-Q; VG Instruments).

RESULTS

The antilisterial substance produced by a lactic acid bacterium isolated from Munster cheese is a bacteriocin. A total of 1,962 bacterial isolates with antagonistic effects on *L. monocytogenes* were isolated from 392 samples of Munster cheese. In most cases, the antagonistic effect was due to a decrease in the pH resulting from the production of organic acids. The culture extracts from only six isolates were shown to be active against *L. monocytogenes* by the action of antibacterial substances other than organic acids. These bacterial isolates were three *Lactococcus* strains, two *Enterococcus* strains, and one *Lactobacillus* strain. The latter proved to be by far the most efficient, with a culture extract activity of 17,070 AU/ml, and as a result was chosen for this study.

The antibacterial substance contained in the culture extract of the *Lactobacillus* strain was not inactivated in the presence of catalase, which excluded an inhibition by hydrogen peroxide. It was however inactivated by all the proteolytic enzymes tested: pepsin, trypsin, α-chymotrypsin, pronase, and ficin. In fact, although the initial culture extract produced an inhibition zone with a diameter of 14 mm in an *L. monocytogenes* 4d layer, no inhibition zone was detected after treatment with these enzymes. The antibacterial substance is, therefore, a substance of a proteinaceous nature. No inactivation was observed in the presence of α-amylase and lipase, which excludes the participation of carbohydrate or lipid moieties in the activity of the antibacterial substance.

The activity spectrum of this substance was relatively narrow, preferentially affecting the closely related species; all *Listeria* species tested were shown to be susceptible to the action of the antibacterial substance (Table 1). In the case of *L. monocytogenes*, all the serotypes tested were susceptible, with inhibition zone diameters ranging from 8 to 14 mm. The activity spectrum also included the strains of other gram-positive bacteria (*Lactobacillus*, *Enterococcus*, *Micrococcus*, *Staphylococcus*, and *Bacillus* spp.). On the other hand, the gram-negative bacteria tested (*Salmonella*, *Pseudomonas*, and *Escherichia* spp.) were not affected by the antibacterial substance.

The substance had a bactericidal mode of action: after only half an hour of incubation in the culture extract of the *Lactobacillus* strain, a reduction of 3 logarithmic units in the concentration of *L. monocytogenes* 4d, initially 2.1×10^7 CFU/ml, was observed. This reduction continued up to 60 min of incubation (bacterial count, 1.5×10^3 CFU/ml) but did so slowly, indicating the exhaustion of the bacteriocin. When the incubation time exceeded 1 h, a resumption in growth of *L. monocytogenes* was observed in the culture extract. Nevertheless, this resumption was very slow (increase of 1 logarithmic unit after 2 h and 2 logarithmic units after 7 h), probably because of the existence of bacterial cellular lesions, which prevented bacteria from dividing rapidly (3).

The bactericidal effect of the bacteriocin produced by the *Lactobacillus* strain was accompanied by bacterial lysis. Scanning electron microscopy of *L. monocytogenes* 4d cells showed that the cells incubated for 30 min with the *Lactobacillus* bacteriocin presented major morphological changes. This was apparently due to changes in the structure of the cell wall (Fig. 1), with the cells rupturing in one or two places and the cell contents escaping. After incubation for 5 h, only the cell wall, showing an irregular and perforated surface, was still visible for cells having undergone lysis (Fig. 1C). This cell wall finally disintegrated, leaving debris that was apparently responsible for the granular appearance of the field of observation.

The study of the chemical nature, the activity spectrum, and the mode of action of the antibacterial substance present in the culture extract of the *Lactobacillus* strain confirmed that this substance was a bacteriocin, according to the definition proposed by Klaenhammer (26).

The amino acid sequence and the primary structure of the bacteriocin isolated are identical to those of the pediocin AcH. The purification steps following the procedure described by

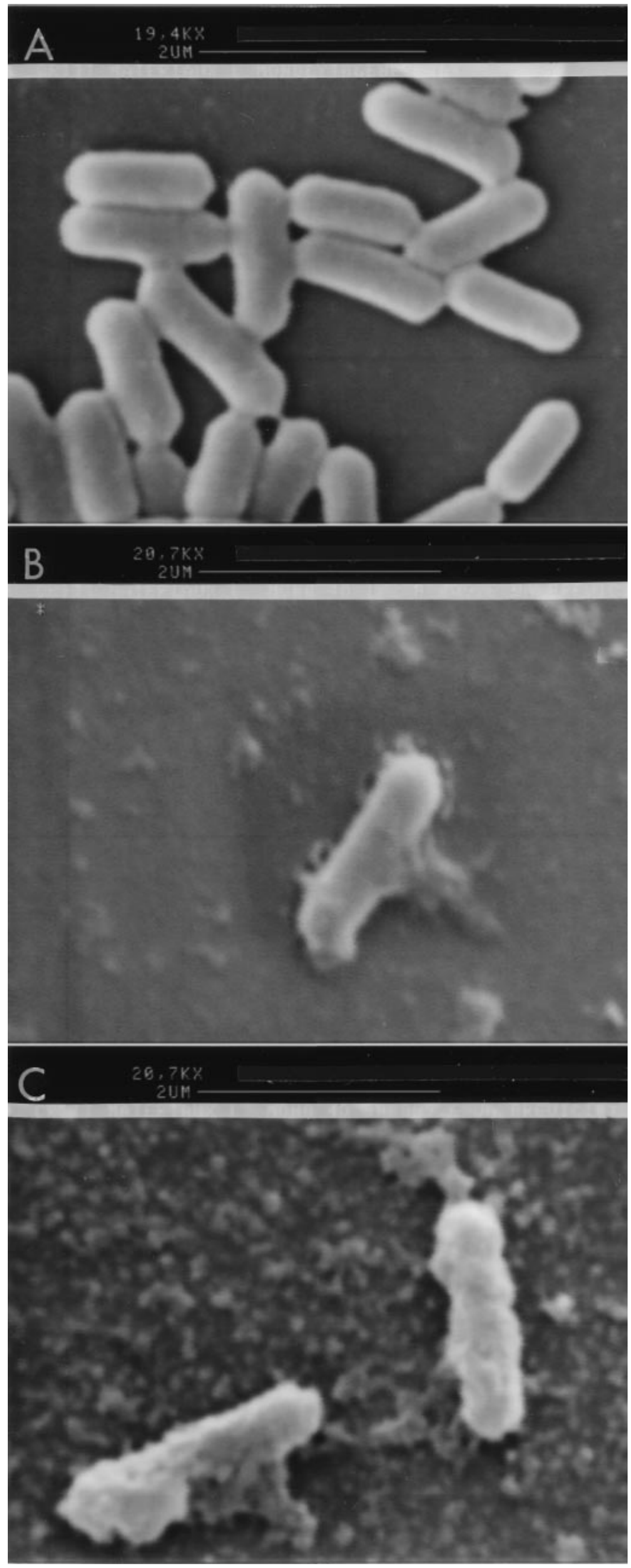

FIG. 1. Scanning electron micrographs of *L. monocytogenes* 4d cells. Cells were left untreated (A) or were treated with the bacteriocin for 30 min (B) or 5 h (C) at 37°C. KX, 19.4, 20.7, and 20.7 for panels A, B, and C, respectively.

TABLE 2. Purification steps of the bacteriocin in this study

Purification stage	Vol (ml)	A_{280}	Total A_{280}[a]	Activity (UA/ml)	Total activity[b] (UA)	Sp act[c]	Yield (%)
Culture extract	2,000	2.00	4,000	1.7×10^4	3.4×10^7	8.5×10^3	100
Fractions							
I (ammonium sulfate precipitation)	250	4.00	1,000	8.5×10^3	2.1×10^6	2.1×10^3	6.2
II (cation exchange)	46	0.46	23.3	3.3×10^4	1.5×10^6	7.2×10^4	4.5
III (octyl-Sepharose)	11	0.42	4.6	3.3×10^3	3.7×10^4	8.0×10^3	0.1
IV (C_2-C_{18} column [injection 2])	1	0.86	0.8	5.3×10^5	5.3×10^5	6.4×10^5	1.6

[a] Total A_{280} is the A_{280} multiplied by the volume (in milliliters).
[b] Total activity is the number of activity units in the fraction volume.
[c] Specific activity is calculated as activity units divided by A_{280}.

Nissen-Meyer et al. (42) are shown in Table 2. The specific activity of the fraction collected after reversed-phase (C_2-C_{18}) chromatography was 14 times higher than that of the fraction obtained after application to the octyl-Sepharose column and 75 times higher than that measured in the culture extract.

A chromatographic peak corresponding to the purified bacteriocin was obtained. The UV absorption spectrum of this molecule indicated the presence of tryptophan residues. The two 1-ml fractions representing the peak, collected and mixed (fraction IV), were used to determine the structure of the bacteriocin. Study of this fraction by mass spectrometry confirmed the purity of the sample and showed the molecular mass of the isolated bacteriocin to be 4,623.60 ± 0.15 Da (mean ± standard deviation). The amino acid sequence of the sample was determined by protein sequence analysis. Edman degradation yielded a clear sequence of 44 amino acids (Fig. 2), which corresponded to a calculated molecular mass of 4,628.2 Da. The difference between the measured molecular mass (4,623.1 Da) and the calculated molecular mass (4,633.2 Da) suggested the existence of two disulfide bonds linking the four cysteine residues on the sequence. Mass spectrometry and Edman degradation were used to confirm and characterize these disulfide bridges (28). Lys-C digestion of the bacteriocin produced two reversed-phase high-performance liquid chromatography peaks, which were measured by electrospray ionization-mass spectrometry and sequenced. A single possibility for the assignment of the disulfide bridges corresponded to masses of the different Lys-C cleavage peptides (2,077.3 Da and 2,471.9 Da). These disulfide bonds were localized between cysteine residues 9 and 14 on one hand and 24 and 44 on the other hand.

A study of the known bacteriocin sequences has shown that the primary structure of the bacteriocin in this study corresponds exactly to that of the pediocin AcH produced by *P. acidilactici* H, for which the existence of disulfide bonds between the same cysteine residues has also been described (36).

The bacteriocin producer strain (designated WHE 92) is an *L. plantarum* strain. The cells were gram-positive rods (data not shown). They were facultatively heterofermentative (gas was produced when the carbon source was gluconate but not when glucose was used). At 37°C, the cells produced acid from ribose, galactose, D-glucose, D-fructose, D-mannose, mannitol, sorbitol, *N*-acetyl glucosamin, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, lactose, sucrose, trehalose, β-gentiobiose, D-arabitol, and gluconate. No acidification occurred with glycerol, erythritol, arabinose, xylose, adonitol, β-methyl-xyloside, L-sorbose, rhamnose, dulcitol, inositol, α-methyl-D-mannoside, α-methyl-D-glucoside, melibiose, inulin, melezitose, D-raffinose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, fucose, L-arabitol, and ketogluconate. These biochemical results suggest that strain WHE 92 may be a strain of *L. plantarum*. To verify this, the genomic DNA from strain WHE 92 was probed with an *L. plantarum* DNA probe in a Southern-type hybridization experiment. In parallel, DNAs from the reference *L. plantarum* strain (CCM 1904) and *P. acidilactici* H, which is the producer of the bacteriocin AcH, were also tested. As can be seen in Fig. 3, three bands, which were the same size as those found with the reference *L. plantarum* strain (lane 3) and differ from the weak band obtained with *P. acidilactici*, were detected with strain WHE 92 (lane 2). Therefore, strain WHE 92 was an *L. plantarum* strain.

The production of pediocin AcH by *L. plantarum* WHE 92 is independent of the final pH of the culture medium between pH 4.0 and pH 6.0. Studies carried out by Biswas et al. (4) have shown that the production of bacteriocin by *P. acidilactici* H decreased significantly when the final pH value of the culture broth exceeded 4.0 and ceased when this value reached 5.5. The latter result has not been confirmed in the present study,

1 10 20

Lys – Tyr – Tyr – Gly – Asn – Gly – Val – Thr – Cys – Gly – Lys – His – Ser – Cys – Ser – Val – Asp – Trp – Gly – Lys

21 30 40

Ala – Thr – Thr – Cys – Ile – Ile – Asn – Asn – Gly – Ala – Met – Ala – Trp – Ala – Thr – Gly – Gly – His – Gln – Gly –

41

Asn – His – Lys – Cys

FIG. 2. Amino acid sequence of the bacteriocin in this study.

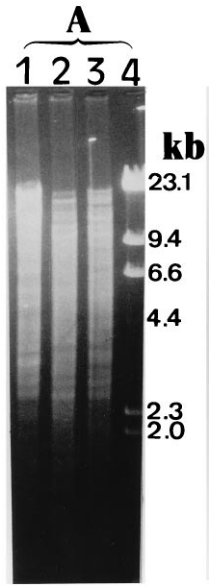

FIG. 3. Southern-type hybridization with an *L. plantarum* DNA probe. (A) Electrophoresis (0.7% agarose gel) of *Bgl*I-restricted genomic DNA. (B) After transfer of the genomic DNAs to a membrane (Hybond-N$^+$; Amersham), the DNAs were probed with the digoxigenin-labelled *pyrDFE* probe from *L. plantarum* CCM 1904 (ATCC 8014) and the hybridized DNA was detected with the nonradioactive DIG DNA labelling and detection kit (Boehringer Mannheim). Lanes 1, *P. acidilactici* H; lanes 2, strain WHE 92; lanes 3, *L. plantarum* CCM 1904; and lanes 4, DNA molecular marker (lambda DNA digested with *Hin*dIII).

carried out with the same strain, since a low-level production of pediocin AcH was still observed when the culture medium was maintained at pH 6.5. Nevertheless, a clear reduction in the quantity of the bacteriocin produced was observed, as in the work of Biswas et al. (4), when the final pH of the culture medium exceeded 4.0 (Fig. 4). The production of pediocin AcH from *L. plantarum* WHE 92 was also tested for pH dependency. The culture extract activity obtained at the end of the exponential growth phase of *L. plantarum* WHE 92 remained constant (68,270 AU/ml), independent of the final pH value of the culture broth between pH 4.0 and 6.0, and decreased only at pH 6.5 (Fig. 4).

Therefore, there is a difference between *L. plantarum* WHE 92 and *P. acidilactici* H concerning the influence of the final culture broth pH on the production of pediocin AcH. This difference cannot be explained by the pH influence on the growth of these bacteria, since good growth of the two bacteria occurred independent of the final culture broth pH between 4.0 and 6.5 (bacterial counts of approximately 2×10^8 to 6×10^9 CFU/ml were always obtained at the end of the exponential growth phase for the two bacteria after inoculation with bacteria to concentrations of 2×10^2 to 4×10^3 CFU/ml).

DISCUSSION

The primary structure of the bacteriocin produced by *L. plantarum* WHE 92, a peptide of 44 amino acid residues, appears to be new among the species *L. plantarum*. In fact, it differs from the primary structures, even partial, of the plantaricins known at this time (8, 16, 22, 24, 43). On the other hand, this structure corresponds exactly to that of pediocin AcH (also known as pediocin PA-1.0 or SJ-1), which is produced by *P. acidilactici* H (36), PAC-1.0 (20), and SJ-1 (48). We report the first case of the same bacteriocin being produced naturally by bacteria of different genera.

It has been shown that the genetic determinant for the production of pediocin AcH was plasmid associated for the three strains of *P. acidilactici* mentioned above (33, 37, 47). The plasmid pSMB74 (8.9 kb [37]), responsible for this production in *P. acidilactici* H, can be transferred intragenerically from one *P. acidilactici* strain to another by conjugation (45). The fact that the plasmid pSQR11, which produces pediocin in *P. acidilactici* PAC-1.0, is by all appearances identical to the plasmid pSMB74 (30, 33) is, therefore, not surprising. The plasmid responsible for the bacteriocin production in *P. acidilactici* SJ-1, the structure of which is still unknown, is, on the other hand, smaller than plasmid pSMB74 (47). An intrageneric transfer, with modification in size, of the plasmid responsible for the bacteriocin production could, however, explain why *P. acidilactici* H and SJ-1 produce the same bacteriocin. Such a transfer has been also observed between strains of *L. lactis* (nisin and other bacteriocins) (6, 13, 15, 18, 41, 50) and *Lactobacillus acidophilus* (lactacin F) (39).

As for *L. plantarum* WHE 92, it appears that the genetic determinant for pediocin production is also plasmid associated. Curing experiments and plasmid profile analysis have suggested the involvement of an 11-kb plasmid with the bacteriocin production by *L. plantarum* WHE 92 (data not shown). Even though the size of the plasmid of *L. plantarum* WHE 92 (11 kb) is different from those of the plasmids involved in the production of pediocin AcH in strains of *P. acidilactici* (30, 33, 37, 47), a modification after acquisition of a conjugative plasmid, analogous to that which could explain the pediocin production by *P. acidilactici* SJ-1, could be envisaged. However, in this case, the plasmid transfer would be intergeneric. Such a transfer has only been shown to occur for nisin determinants from *L. lactis* to *Leuconostoc dextranicum* (55); this would be the first case of native intergeneric conjugal transfer of bacteriocin determinants.

By using the broad-host-range resistance plasmid pIP501, conjugative transfer events have been shown to occur from *Pediococcus* to *Lactococcus* spp. (17) and from *Lactococcus* spp. to *L. plantarum* (60). Furthermore, numerous studies have clearly affirmed the conjugative potential of members of the family *Lactobacillaceae* (14, 46, 59, 60). Therefore, genetic

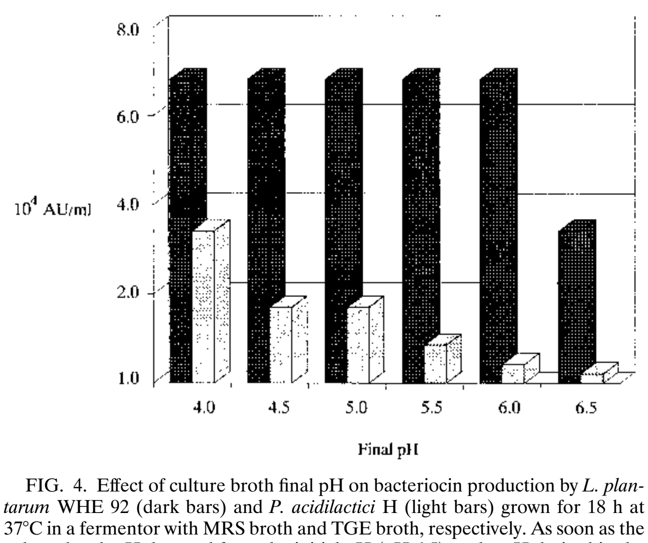

FIG. 4. Effect of culture broth final pH on bacteriocin production by *L. plantarum* WHE 92 (dark bars) and *P. acidilactici* H (light bars) grown for 18 h at 37°C in a fermentor with MRS broth and TGE broth, respectively. As soon as the culture broth pH dropped from the initial pH (pH 6.5) to the pH desired in the study (final pH), it was automatically maintained by adding sterile 1.0 M NaOH. At the end of the incubation, culture extracts were taken and bacteriocin production was monitored by titration.

transfer by conjugation between *Pediococcus* and *Lactobacillus* spp. could be considered.

According to several authors (23, 44, 61), the posttranslational processing of the prepediocin to active pediocin AcH by *P. acidilactici* H can take place efficiently only when the pH of the culture medium is less than or equal to 5.0. This would explain why the production of pediocin AcH from this bacterium decreases when the final pH of the culture medium exceeds this value (see above). Assuming that the genetic organization of the pediocin AcH production from *L. plantarum* WHE 92 is analogous to that observed for the pediococci (33, 37) and for many class II-bacteriocin-producing lactic acid bacteria (1, 8, 51, 56, 57), the posttranslational processing of the prepediocin to pediocin in *L. plantarum* WHE 92 and *P. acidilactici* H would take place differently, since no reduction in the pediocin AcH production from *L. plantarum* WHE 92 is observed up to pH 6.0. However, these different hypotheses could only be investigated with the study of the structure of the 11-kb *L. plantarum* WHE 92 plasmid.

The pediococci involved in pediocin AcH production have not been used to combat *L. monocytogenes* in dairy products. In fact, these bacteria are poorly adapted to this foodstuff category in which they are not naturally present (38, 49). The importance of the problem posed by the contamination of dairy products with *L. monocytogenes* (11) has, moreover, led Marugg et al. (33) to envisage the transfer of the pediocin AcH production capacity from *P. acidilactici* to lactococcal strains in order to use these bacteria in contaminated dairy products. On the other hand, *L. plantarum* WHE 92 develops particularly well in cheeses, especially those of the Munster type, in which bacterial counts of 10^8 CFU/g have been found. Additionally, *L. plantarum* constitutes a predominant species amongst the contaminating bacteria (secondary flora) normally present in milk curds, which play an important part in the ripening of certain cheeses (35). In other respects, the level of pediocin AcH production from *L. plantarum* WHE 92 appeared quantitatively higher (under optimum culture conditions) and above all much less dependent on the final pH of the culture medium than did that from *P. acidilactici* H. More precisely, the fact that no reduction in the production of pediocin AcH from *L. plantarum* WHE 92 was observed up to pH 6.0 (whereas its production from *P. acidilactici* H was considerably reduced when the final pH of the culture medium exceeded 5.0) is particularly important since the pH of dairy products is often higher than 5.0. The industrial utilization of *L. plantarum* WHE 92 in cheeses susceptible to contamination by *L. monocytogenes*, therefore, seems altogether possible.

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